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Reciprocal Expression of *ospA* and *ospC* in Single Cells of *Borrelia burgdorferi*▽

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Outer surface proteins (Osp) A and C of the Lyme disease spirochete (*Borrelia burgdorferi*) are selectively produced and of functional significance in the tick vector and mammalian host, respectively. Some studies indicate a simple, reciprocal relationship where the signals and pathways that turn on *ospC* also turn off *ospA*. Other studies indicate a more complex regulation where many spirochetes produce both proteins and others produce one of the proteins or neither protein. Here, we have used flow cytometry to characterize *ospA* and *ospC* transcript and protein levels in individual bacterial cells grown in culture. The results support a simple, reciprocal model where, at the level of single cells, the transcription of *ospC* is linked to the repression of *ospA*. We also demonstrate that under conditions conducive for OspC production, spirochetes display an "all or none" response, with some cells displaying high levels of *ospC* transcription and others demonstrating little or no transcription. Despite the reciprocal regulation of *ospA* and *ospC* at the single-cell level, we propose that spirochetes display an array of phenotypes due to stochasticity in the pathways that regulate *osp* expression and the slow turnover of outer surface proteins.

Borrelia burgdorferi, the spirochete responsible for Lyme disease, is transmitted by *Ixodes* ticks (3). *B. burgdorferi* persistently colonizes the gut of ticks. When infected ticks feed, the spirochetes multiply within the gut, migrate to the tick's salivary glands, and infect the vertebrate host (1). Within the feeding tick, the spirochetes alter the expression of many genes in preparation for transmission and infection of the new host (1, 26). *B. burgdorferi* outer surface proteins (Osp) A and C have served as a paradigm for understanding the regulation of bacterial gene expression within feeding ticks. In ticks, *ospA* is predominantly expressed before the blood meal, whereas *ospC* is induced during the blood meal (9, 17, 22, 23). The functions of these two proteins are consistent with their pattern of expression, where OspA is required for colonizing the vector and OspC is required for infecting the host (12, 19, 29).

Temperature, pH, and cell density act as signals for regulating the expression of *ospA* and *-C* in culture, and these signals are likely to play a role in the feeding tick as well (26). Proteomic and microarray studies with cultured spirochetes grown in "tick-like" (low temperature, high pH) or "host-like" (high temperature, low pH) conditions have led to the identification of large subsets of *Borrelia* proteins and genes with "OspA-like" or "OspC-like" patterns of expression (18, 20). The bacterial signaling pathway regulating the expression of *ospC* and *ospC*-like genes has been characterized in some detail (6, 7, 14, 24, 27, 28). The pathway is activated by a two-component system consisting of a sensor with a histidine kinase domain (HK2) and a cytoplasmic response regulator protein (Rrp2) (14, 27). Activated Rrp2, together with the alternative sigma factor RpoN, induces the expression of many genes, including a second alternative sigma factor, RpoS (24). RpoS activates the transcription of *ospC* and *ospC*-like genes associated with tick transmission and host infection (6, 7, 10).

The pathways and signals regulating *ospA* and *ospA*-like genes expressed in the vector are not as well characterized as the *ospC* expression signaling pathway. Some studies indicate a simple reciprocal relationship where the signals and pathways that induce *ospC* expression within feeding ticks also repress *ospA* expression (21). Studies also indicate that even under ideal conditions for *ospC* expression, the bacterial population is heterogeneous, with many spirochetes producing both proteins and others producing either one or neither of the two proteins, indicating a more complex regulation of *ospA* and *-C* (13, 17). We have developed flow cytometry as a method for following the phenotypes of individual spirochetes and applied this method to better understand the regulation of *ospA* and *ospC*.

MATERIALS AND METHODS

Borrelia strains and culture conditions. A low passage culture of *B. burgdorferi* strain B31 (originally isolated from a tick in Shelter Island, NY) was provided to us by the Centers for Disease Control and Prevention, Fort Collins, CO (4). The stock was cloned on solid Barbour-Stoenner-Kelly H (BSK-H) medium and named B31-C1. Strains A3ntrA-Gm (*rpoN*-null mutant) and A3-Gm (control strain for *rpoN*-null mutant) were obtained from Frank Gherardini, NIAID, Rocky Mountain Laboratories, Hamilton, MT (10). Strains B31-CGFP and B31-FGFP were obtained from James Carroll, University of Pittsburgh, Pittsburgh, PA (8). B31-CGFP is B31 clone A3 harboring the plasmid pBSVΦ(*ospCp-gfp*), and B31-FGFP is B31 clone A3 harboring the plasmid pBSVΦ(*flaBp-gfp*). Plasmid pBSVΦ(*ospCp-gfp*) has *gfp* under the control of the *ospC* promoter, whereas in plasmid pBSVΦ(*flaBp-gfp*), *gfp* is under the control of the *flaB* promoter. Cultures were grown in BSK-H complete medium (Sigma, St Louis, MO). The culture was grown at 35°C until the cells reached a density of 1×10^5 cells/ml. Cells were then transferred into fresh BSK-H complete medium for experiments.

Direct fluorescence antibody staining and flow cytometry analysis of *Borrelia*. To stain spirochetes, fluorescently labeled monoclonal antibodies (MAbs) against OspA (C3.78 MAb-Alexa488) and OspC (B5 MAb-Alexa647), provided by Fred Kantor (Yale University) and Lamine Mbow (CDC, Fort Collins, CO), respectively, were used (11, 15). *B. burgdorferi* cultures were harvested and incubated at room temperature for 30 min with anti-OspC and anti-OspA MAbs

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TABLE 1. Measurement of *ospC* transcript levels in a phenotypically distinct subpopulation of *B. burgdorferi* B31-C1[a]

Cell phenotype	Avg C_T of:		ΔC_T *ospC-flaB*[b]	$\Delta\Delta C_T$[c]	Normalized *ospC* amt relative to $OspA^+$ $OspC^-$ cells[d]
	ospC	*flaB*			
$OspA^+/OspC^+$	30.59 ± 0.18	28.69 ± 0.47	1.90 ± 0.50	−4.74 ± 0.50	26.8 (18.8–38.1)
$OspA^-/OspC^+$	28.54 ± 0.22	29.07 ± 0.37	−0.53 ± 0.43	−7.18 ± 0.43	145.51 (107.68–196.63)
$OspA^+/OspC^-$	36.21 ± 0.29	29.56 ± 0.41	6.64 ± 0.50	0.00 ± 0.50	1.0 (0.70–1.42)

[a] *Borrelia* cultures grown at 5×10^7 cells/ml were used for sorting by flow cytometry and subsequent quantitative RT-PCR analysis.
[b] The ΔC_T value was determined by subtracting the average *flaB* threshold cycle (C_T) value from the average *ospC* C_T value. The standard deviation of the difference is calculated from the standard deviations of the *ospC* and *flaB* values.
[c] The calculation of $\Delta\Delta C_T$ involves subtraction of the ΔC_T calibrator value from the ΔC_T value [$\Delta C_T - \Delta C_{T(OspA^+\ OspC^-)}$]. This is subtraction of an arbitrary constant, so the standard deviation of $\Delta\Delta C_T$ is the same as the standard deviation of ΔC_T.
[d] Ranges (in parentheses) were determined by evaluating the expression $2^{-\Delta\Delta C_T}$ with $\Delta\Delta C_T + s$ and $\Delta\Delta C_T - s$, where s is the standard deviation of the $\Delta\Delta C_T$ value.

at room temperature. Labeled spirochetes were washed twice with phosphate-buffered saline (PBS) and analyzed on a MoFlo modular flow cytometer (Cytomation, Inc., Fort Collins, CO) with a 15-mW, 488-nm argon ion laser and a Coherent Innova-90 krypton laser at 350 to 360 nm. Data were acquired and analyzed using Summit V.3.1 (Cytomation, Inc., Fort Collins, CO).

Quantitative RT-PCR for *ospA* and *ospC* mRNA. A culture was grown at 35°C to a density of 5×10^7 bacteria/ml. Approximately 10^6 *Borrelia* cells were sorted by flow cytometry into A^+/C^-, A^+/C^+, and A^-/C^+ populations. The sorted cells were added to a buffer containing PBS, 100 mM dithiothreitol, and RNase inhibitor (RNAsin; Promega). Cells were subjected to four freeze-thaw cycles. Genomic DNA in the cell lysates was removed using a Turbo DNA-free kit (Ambion). Reverse transcription (RT) was performed using random primers (Invitrogen). The cDNA was used for quantitative PCR using Sybr green master mix (Applied Biosystems, Foster City, CA). PCR was performed on an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA), using the following specific primer pairs: flaB-F (TTTCAGGGTCTCAAGCG TCT) and flaB-R (TGTTGAGCTCCTTCCTGTTG), ospC-F (GAAAGAGGT TGAAGCTTGC) and ospC-R (ATTGCATAAGCTCCCGCTAA), and ospA-F (GCAGCCTTGACGAGAAAAAC) and ospA-R (GGATCTGGAGTACTTG AAGGC). The thermal conditions applied for amplification were 1 cycle at 95°C for 15 min and 50 cycles at 95°C for 15 s and 60°C for 1 min. Melting curves were generated by treating the amplified samples at 95°C for 15 s, 60°C for 20 s, and 95°C for 15 s. Transcript levels of *ospA* and *ospC* in these cell populations were calculated by the $2^{-\Delta\Delta CT}$ method (16) (Tables 1 and 2).

RESULTS

OspA and OspC phenotypes of individual spirochetes grown in culture. Experiments were done to characterize OspA and OspC production by individual bacterial cells at different temperatures and cell densities. Strain B31-C1 cultures were started at a density of 1×10^3 bacteria/ml and grown at 35°C until the cultures reached densities of 1×10^6, 1×10^7, and 5×10^7 bacteria/ml. A control culture was grown at 23°C to a density of 5×10^7 cells/ml. Spirochetes were stained with anti-OspA C3.78 MAb-Alexa488 and anti-OspC B5 MAb-Alexa647 and analyzed by flow cytometry. *Borrelia* cells were plotted, with OspA staining intensity on the *x* axis and OspC staining intensity on the *y* axis. The plot was divided into four quadrants, representing A^+/C^+, A^+/C^-, A^-/C^+, and A^-/C^- cells (Fig. 1).

The control culture grown at 23°C and the culture grown at 35°C to a density of 1×10^6 bacteria/ml consisted almost exclusively (99%) of A^+/C^- bacteria (Fig. 1A and B). As the culture grew from a density of 1×10^6 to 5×10^7 cells/ml at 35°C, the number of bacteria producing only OspC increased from 0.1 to 24%, whereas the proportion of bacteria producing only OspA decreased from 99 to 62% (Fig. 1B to D). The shapes of the scatter plots indicate that the spirochetes first shifted to a population containing both OspA and OspC, and these double-positive bacteria further increased expression of OspC while decreasing levels of OspA (Fig. 1B to D). These results demonstrate that the cells that increase OspC are the same cells that decrease OspA.

***ospC* and *ospA* mRNA levels in spirochete populations with distinct phenotypes.** A *Borrelia* culture was grown to 5×10^7 cells/ml and sorted by flow cytometry to isolate A^+/C^-, A^+/C^+, and A^-/C^+ populations. We used quantitative RT-PCR to estimate *ospA* and *ospC* transcript levels in each population. When *ospC* transcript levels were compared among the three populations, the double-positive spirochetes (A^+/C^+) and the spirochetes that produced only OspC (A^-/C^+) were found to have 26.8- and 145.5-fold more *ospC* transcripts, respectively, than the spirochetes that did not produce OspC (A^+/C^-) (Table 1). When *ospA* transcript levels were compared among the three populations, the double-positive spirochetes (A^+/C^+) and the spirochetes that produced only OspA (A^+/C^-) were found to have 2.6- and 7.34-fold more *ospA* transcripts, respectively, than the spirochetes that did not produce OspA ($A^-/$

TABLE 2. Measurement of *ospA* transcript levels in a phenotypically distinct subpopulation of *B. burgdorferi* B31-C1[a]

Cell phenotype	Avg C_T of:		ΔC_T *ospA-flaB*[b]	$\Delta\Delta C_T$[c]	Normalized *ospA* amt relative to $OspA^-$ $OspC^+$ cells[d]
	ospA	*flaB*			
$OspA^+/OspC^+$	27.29 ± 0.22	28.69 ± 0.47	−1.39 ± 0.52	−1.39 ± 0.52	2.6 (0.48–1.52)
$OspA^-/OspC^+$	29.07 ± 0.20	29.07 ± 0.37	−0.007 ± 0.42	0.00 ± 0.42	1.0 (0.74–1.33)
$OspA^+/OspC^-$	26.68 ± 0.12	29.56 ± 0.41	−2.88 ± 0.43	−2.87 ± 0.43	7.34 (5.44–9.92)

[a] *Borrelia* cultures grown at 5×10^7 cells/ml were used for sorting by flow cytometry and subsequent quantitative RT-PCR analysis.
[b] The ΔC_T value is determined by subtracting the average *flaB* threshold cycle (C_T) value from the average *ospA* C_T value. The standard deviation of the difference is calculated from the standard deviations of the *ospA* and *flaB* values.
[c] The calculation of $\Delta\Delta C_T$ involves subtraction of the ΔC_T calibrator value from the ΔC_T value [$\Delta C_T - \Delta C_{T(OspA^-\ OspC^+)}$]. This is subtraction of an arbitrary constant, so the standard deviation of $\Delta\Delta C_T$ is the same as the standard deviation of ΔC_T.
[d] Ranges were determined by evaluating the expression $2^{-\Delta\Delta C_T}$ with $\Delta\Delta C_T + s$ and $\Delta\Delta C_T - s$, where s is the standard deviation of the $\Delta\Delta C_T$ value.

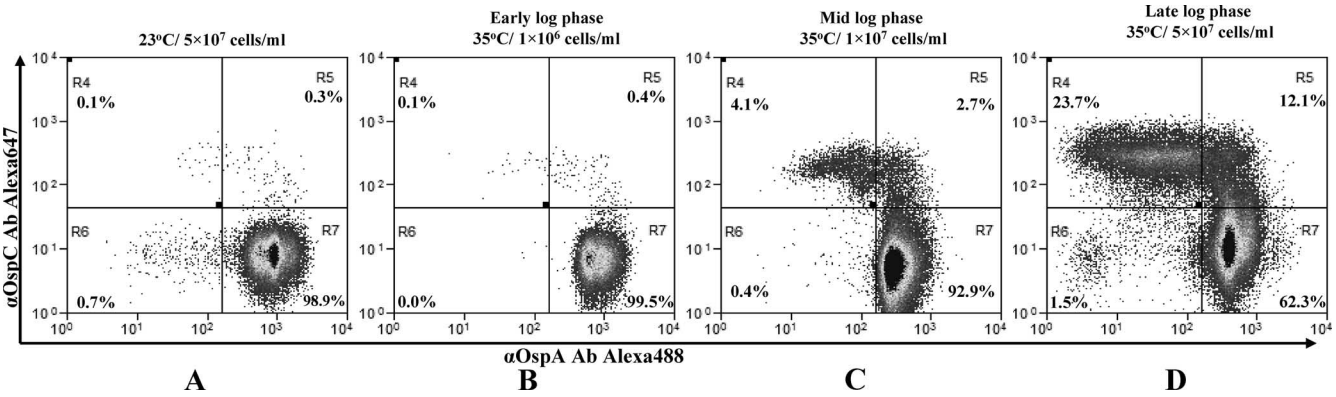

FIG. 1. Outer membrane phenotypes of *B. burgdorferi* grown to different cell densities. Strain B31-C1 was grown at 23°C to a density of 5×10^7 cells/ml (A) or at 35°C to densities of 1×10^6 cells/ml (B), 1×10^7 cells/ml (C), and 5×10^7 cells/ml (D); stained with anti-OspA (αOspA) C3.78 MAb-Alexa488 and anti-OspC B5 MAb-Alexa647 antibodies; and analyzed by fluorescence-activated cell sorting (FACS). Results shown are representative of three independent experiments. The quadrants in each plot were drawn to represent subpopulations that are $OspA^-/OspC^+$ (R4), $OspA^+/OspC^+$ (R5), $OspA^-/OspC^-$ (R6), and $OspA^+/OspC^-$ (R7).

C^+) (Table 2). These results indicate that even the double-positive population consists of bacteria that are in the process of up-regulating *ospC* and down-regulating *ospA*. Based on these results, we interpret the double-positive phenotype as a transient phenotype most likely caused by the stability of pre-existing OspA protein.

Role of RpoN in OspA down-regulation. If *ospA* and *ospC* are reciprocally regulated in single spirochetes, it is logical to assume that the RpoN/RpoS pathway that induces *ospC* and *ospC*-like genes also represses *ospA* and *ospA* like genes (6, 7, 14, 24, 27, 28). To directly test the role of RpoN in *ospA* expression, we used wild-type (B31-A3 WT) and *rpoN* mutant (B31-A3 Δ*rpoN*) strains created by Fisher et al. (10) in flow cytometry experiments. The bacteria were cultured at 35°C to late log phase (5×10^7 cells/ml), stained with anti-OspA MAb-Alexa488 and anti-OspC MAb-Alexa647, and then analyzed by flow cytometry. As expected, B31-A3 Δ*rpoN* did not produce any OspC, as RpoN is required for *ospC* expression (data not shown) (5, 14). When the wild-type and Δ*rpoN* mutant strains were examined for loss of OspA, 15.61% of B31-A3 WT and 1.21% of the Δ*rpoN* strain were OspA negative (Fig. 2). These results demonstrate that RpoN is required for the induction of *ospC* as well as for the repression of *ospA* observed in culture.

Binary distribution of OspC-positive and -negative spirochetes. The flow cytometry data indicate that even under optimal conditions for OspC production, only a subpopulation of spirochetes induces the protein (Fig. 1D). To further understand the nature of this phenomenon, we used flow cytometry to measure the mean fluorescence intensity of OspC in individual cells grown at 35°C to a density of 5×10^7 cells/ml. The results were expressed by plotting mean fluorescent intensity on the *x* axis and bacterial cell count on the *y* axis (Fig. 3). Even under optimal conditions for OspC production, a small population of cells (19.11%) expressed a high level of OspC (mean fluorescent intensity = 306.06) while the majority of cells expressed low levels of OspC (Fig. 3C), comparable to expression levels found in spirochetes grown under conditions unfavorable for OspC production (mean fluorescent intensity = 11.05) (Fig. 3A and B). A similar "all or none" response was observed when we measured transcription from the *ospC* promoter by using B31-A3 strains created by Carroll et al. containing *gfp* fused to a *flaB* or *ospC* promoter (8). In *Borrelia* strains containing *gfp* fused to the *ospC* promoter, 5.14% of cells expressed high levels of green fluorescent protein (GFP) (mean fluorescent intensity = 63.13), while the rest of the cells in the population expressed negligible amounts of GFP (mean fluorescent intensity = 2.00) (Fig. 3D). Almost all the *Borrelia* cells containing GFP under the *flaB* promoter expressed high levels of GFP (mean fluorescent intensity = 31.12) (data not shown). These results demonstrate that *ospC* transcription is regulated by an "all or none" response at the level of individual bacterial cells.

DISCUSSION

Flow cytometry is a powerful technique that can be used to study heterogeneity between cells in a population. Here, we

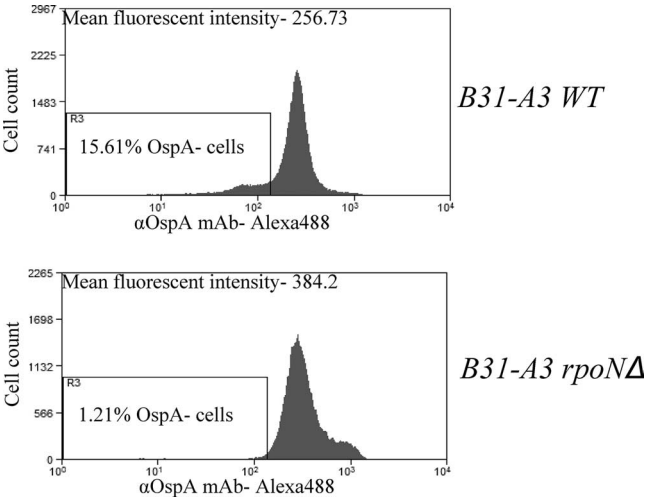

FIG. 2. RpoN is required for the down-regulation of OspA in *B. burgdorferi*. Wild-type (B31-A3 WT) and *rpoN* mutant (B31-A3 Δ*rpoN*) strains were grown at 35°C to a density of 5×10^7 cells/ml. Cultures were stained with anti-OspA (αOspA) C3.78 MAb-Alexa488 and analyzed by FACS.

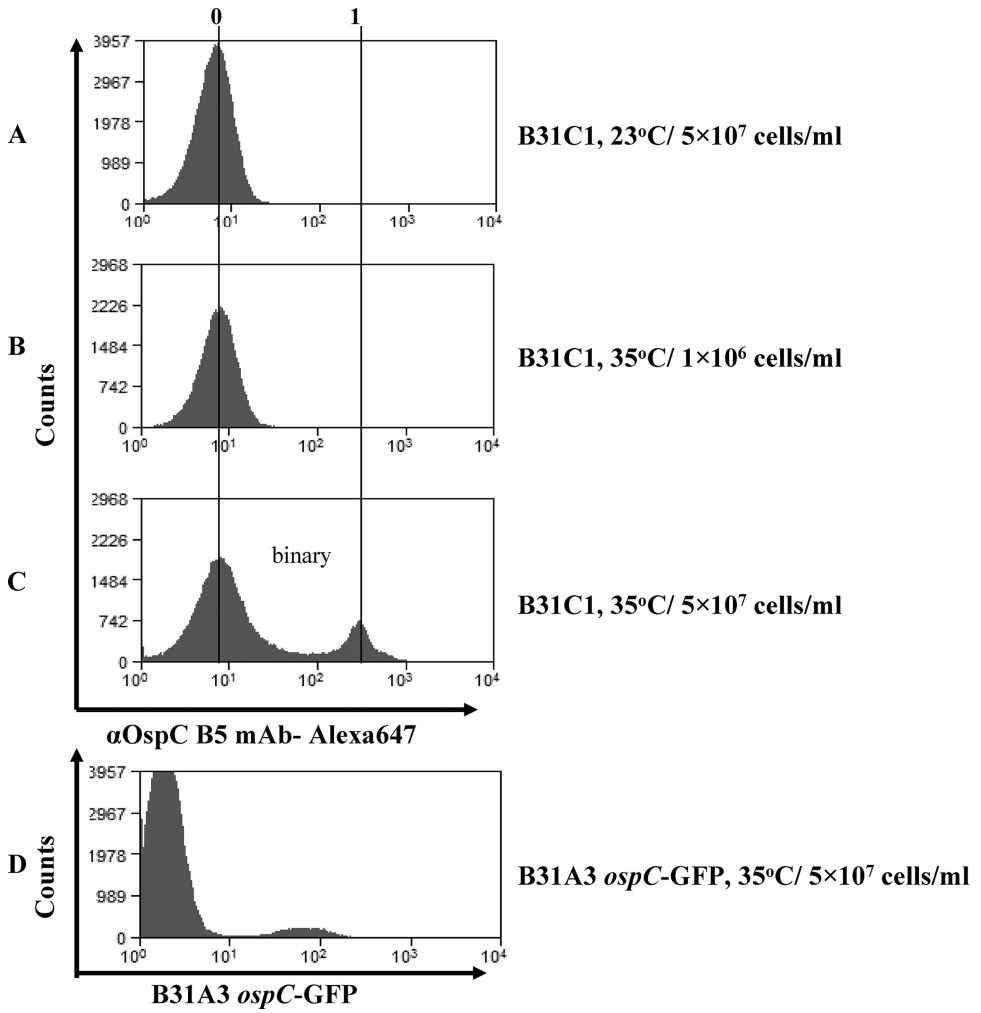

FIG. 3. *ospC* expression is binary. Strain B31-C1 was grown at 23°C to a density of 5×10^7 cells/ml (A) or at 35°C to densities of 1×10^6 cells/ml (B) and 5×10^7 cells/ml (C). Strain B31A3 *ospC*-GFP, containing *gfp* fused to the *ospC* promoter, was grown at 35°C to a density of 5×10^7 cells/ml (D). To measure OspC levels in individual spirochetes (A, B, C), the bacteria were stained with anti-OspC (αOspC) B5 MAb-Alexa647 and analyzed by FACS. Transcriptional activity from the *ospC* promoter (D) was determined by using FACS to measure GFP levels. The data are plotted to display the level of OspC or GFP on each spirochete on the *x* axis and the number of cells on the *y* axis. Note that under conditions conducive for *ospC* expression, individual cells display a binary "all or none" response instead of a graded response.

have used this tool to understand the regulation of *B. burgdorferi ospC* and *ospA*. Most studies for characterizing *ospA* and *ospC* transcript and protein levels under different culture conditions have been done by analyzing the total protein and RNA levels in the culture. An assumption made in these experiments is that all bacteria in the culture respond in similar manners to signals that regulate gene expression. Our results demonstrate that even in a culture, where all spirochetes experience the same temperature, pH, and cell density, *ospC* expression is binary, with some bacteria producing high levels of the protein and others producing little or no protein. Even for in vitro conditions that enhance OspC production (high temperature and high cell density), our results demonstrate that the population consists of two subpopulations with or without OspC. The studies with the o*spC* promoter-GFP fusion establish that the binary response is regulated at the level of transcription.

Our results also demonstrate that, within individual spirochetes, *ospA* and *ospC* are reciprocally regulated by the RpoN/RpoS pathway. The spirochetes that increase OspC are the same ones that down-regulate OspA. In these cells, OspC protein levels increase rapidly, while OspA protein levels decrease more slowly, most likely because of the stability of preexisting OspA. This gives rise to an intermediate A^+/C^+ population from which the spirochetes that produce only OspC emerge as the OspA protein is degraded (Fig. 1). We conclude that heterogeneous populations of spirochetes are found due to the stability of presynthesized protein and the nonresponsiveness of some bacteria. However, individual spirochetes that change the expression patterns of *ospC* and *ospA* do so in a reciprocal manner where the RpoN/RpoS pathway activates *ospC* and represses *ospA* transcription. This conclusion is supported by a recent study (6) demonstrating that RpoS is required for *ospC* induction and *ospA* repression in vivo.

Many *Borrelia* genes are differentially expressed and function at a specific stage in the life cycle. There is much interest in defining the signals and mechanisms regulating gene expres-

sion in *Borrelia.* Our discovery about the binary "all or none" expression of *ospC* at the level of individual cells adds another layer of complexity to our current understanding of *Borrelia* gene expression. It is now well established that many microbes, especially vector-borne pathogens, display phenotypic heterogeneity even within a genetically homogenous culture (2). Stochasticity and epigenetic regulation have emerged as major mechanisms driving cell-to-cell variation in the absence of genetic variation (2). We propose that stochasticity in the pathway that regulates *ospC* expression accounts for the "all or none" binary response observed for OspC in this study. Once a cell enters the "on" state, a positive-feedback loop could maintain high-level expression (25). Since *ospC* expression involves two global gene expression regulators, RpoN and RpoS, we predict that many other genes will also display the binary pattern described here for *ospC*. This phenotypic heterogeneity is likely to help the *Borrelia* cell adapt to the different, ever-changing environments encountered during its complex life cycle in the vector and host.

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